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Analysis of Ductal Lavage Fluid

PRINCIPAL INVESTIGATOR: Saraswati Sukumar, Ph.D.

CONTRACTING ORGANIZATION: John Hopkins University School of Medicine
Baltimore, Maryland 21205

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Introduction:

Detected early, breast cancer is an eminently curable disease. However, reliable intermediate biological markers for breast cancer risk, that can be easily detected in both pre- and post-menopausal women, do not exist at the present time. Conventional cytological examination of breast cells is a fairly reliable test to detect breast cancer cells. However, its sensitivity and specificity could be complemented and supplemented with molecular diagnostic tests. It is increasingly clear that silencing of gene expression by promoter hypermethylation is a common feature of cancer and is seldom seen in normal tissues except for imprinted genes and genes on the inactive X chromosome. Using a sensitive assay called methylation specific PCR (1), initial studies show that MSP can detect 1 methylated gene copy in 1000 unmethylated gene copies, attesting to the sensitivity of this approach. Importantly, the assays are highly specific in that no abnormal methylation was detected in serum DNA if the same alteration was not present in the primary tumor. Promoter methylation has been reported in 15-50% of primary breast tumors for the following genes: 14.3.3 sigma (2,3), RAR-beta (4, 5), cyclin D2 (6), HOXA5 (7), Twist (8), RASSF1A (9, 10, 11), HIN-1 (12) and NES-1 (13), to name a few.

Body:

Month 1 to 6: Optimize conditions for duplex or multiplex assays for the 5 markers RAR β , RASSF1A, Twist, cyclin D2, and HIN-1 using fluid spiked with varying numbers of tumor cells.

We standardized quantitative methylation specific PCR (14) for all five genes. Here the readout is of the percentage of methylation present in each sample, and one can calculate how many microgram equivalents of methylated DNA are present in the particular sample. This method is able to detect down to 20 picogram of methylated DNA in vast excess of unmethylated DNA from normal cells. However, DNA recovered from these samples is a limiting factor, which we have now addressed by developing a multiplex MSP assay.

Then test the MSP markers on ductal lavage from tumor-containing breast of 25 women just prior to surgery for known lesion.

Ductal lavage was performed on 11 women so far in the IRB approved trial on ductal lavage, just prior to surgery for a biopsy proven lesion. These fluids are currently undergoing cytology analysis. Once the analysis is complete, DNA will be extracted from the cytospin preparations and tested for the presence of methylated genes.

Months 7 to 12: If sample is limiting, perform RAR β , RASSF1A, Twist, cyclin D2, and then HIN-1 in a stepwise fashion, going from the highest (85%) to the lowest (30%) incidence markers. Samples may need pre-amplification to enable use of all 5 markers on the varying numbers of tumor cells obtained by ductal lavage. Standardize this methodology. Then test the MSP markers on ductal lavage from tumor-containing breast of 25 additional women just prior to surgery for known lesion.

We have made considerable progress on this specific aim. Knowing that cells from ductal lavage will always remain a limiting factor, we have developed a multiplex quantitative Methylation specific PCR. This PCR test allows us to test all 5 genes with little limitation of sample size. It is important to ascertain whether the increase in sensitivity of this method does not result in loss of specificity. Also, we would like to set the normal range of values from normal tissue, thus setting the cut-off value above which the values can be considered abnormal. This has been completed for each gene using reduction mammoplasty specimens. Also to determine if epithelial or stromal cells provide false positive values for some genes, we microdissected cells from sections of benign and normal breast sections and performed the same assay. The cut-off values have been set at the higher limit of 1% methylation as being normal values.

We plan to use a novel quantitative multiplex methylation specific PCR (MQ-MSP) assay, recently developed by us, on cells from the fixed and stained cytospin preparation to detect 5 genes on DNA obtained from the ductal cells of breast cancer patients. The five genes, Cyclin D2, RAR- β , Twist, RASSF1A, and HIN-1, were selected because they are specifically and frequently (more than 30%) hypermethylated in breast tumor cells, but are not methylated in normal blood cells or normal breast cells. Actin is the 6th gene which is amplified as an internal control for normalization of the reactions based on total DNA content. Multiplex Q-MSP test is very specific, is conducted in a multi-well format suited to large-scale analysis, and provides finite values for the amount of methylated DNA present in each sample as the reaction is progressing in real time (our unpublished data). More importantly, the test is objective, and its sensitivity is well established. The panel of these 5 markers can detect breast cancer (as early as DCIS) in 100% of women (our unpublished data). Most tumors contain 1 or more markers hypermethylated. Combining the sensitivity and objectivity of the test with the specificity of the markers, we have a good chance of identifying women at high risk of developing breast cancer.

Standardization of the Multiplex Q-MSP.

Quantitative real-time -MSP (Q-MSP) test is very specific, is conducted in a multi-well format suited to large-scale analysis, and provides values for the relative amount of methylated and unmethylated DNA present in each sample as the reaction is progressing (in real time). More importantly, unlike conventional MSP (1), the test is objective, and its sensitivity is well established.

We have standardized this assay in a multiplexed format for 6 genes. Using only one aliquot (out of 10 μ l) of the sodium bisulfite treated DNA, in Step 1 all the genes in the panel are amplified in one tube. The first reaction contained 6 gene-specific "external" primers that were designed to anneal to genomic DNA independent of methylation status of the genome (do not contain any CpGs in their own sequence). In step 2, this DNA is diluted, and subjected to Q-MSP for both methylated and unmethylated sequences, using 3 gene-specific primers (2 flanking primers and 1 probe) for the CpG containing region. We have standardized the analysis for the RASSF1A gene in Step 2. We determined that human sperm DNA (HSD) is unmethylated (100%), and that MDA MB 231 breast cancer cell line DNA is methylated (100%) at the RASSF1A promoter (Figure 1A). Human sperm DNA and 231 DNA was mixed in roughly equal proportions. 20 ng of the mixture

DNA was amplified by 35 cycles in a 25 microliter PCR reaction using forward and reverse primers for RASSF1A, RAR β , Cyclin D2, Twist, Hin-1, and actin (6 pairs of primers used simultaneously) in the multiplex PCR reaction. This DNA was then used as a source of the "standard".

To provide a quantitative estimate of the amount of methylation present in each sample, the following was done. In each run, the multiplexed DNA "standard" was serially diluted and amplified with RASSF1A primers specific for either methylated or unmethylated DNA. By plotting Ct vs dilution, a linear trend line with a correlation coefficient $>.99$ was established. It is also established that the slope of the U curve and M curve are approximately the same, indicating both reactions are occurring with the same efficiency (e.g. 2 fold increase per cycle, 10 fold increase every 3.3 cycles). DNA dilution equivalents in the test samples were extrapolated off the standard curve for 20 ng HSD and 231 genomic DNA. % Methylated DNA is calculated by-

$$\% \text{ Methylated DNA} = [M/(U+M)]100$$

Figure 1A

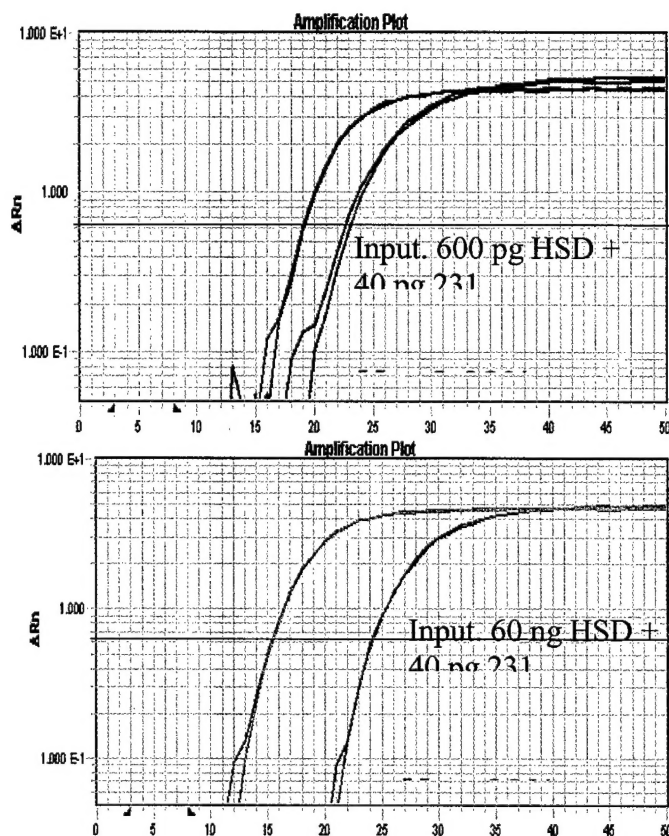


Figure 1B

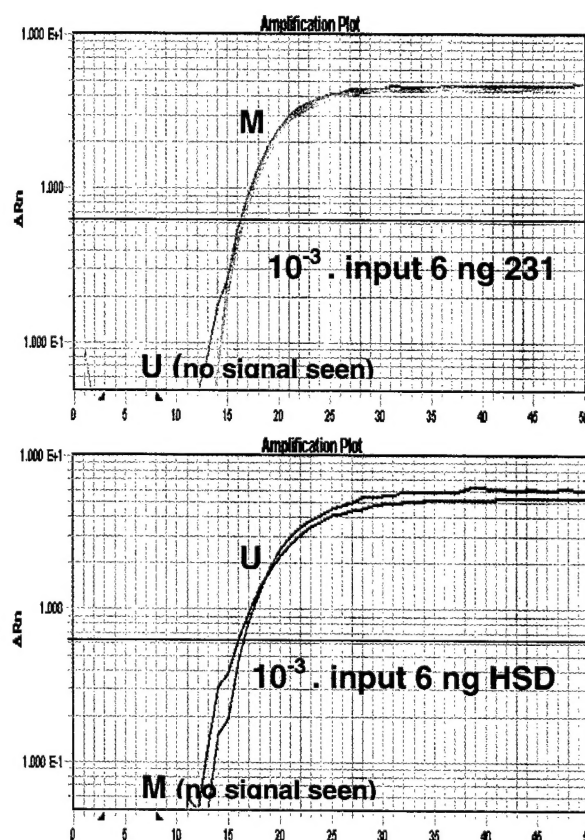


Figure 1A. M-Q-MSP is sensitive. Panel A: Multiplexed DNA (mixture) was diluted (10-3) and Q-MSP was performed using primers specific for the unmethylated (U) or methylated (M) RASSF1A promoter. Panel A: 600 pg of HSD plus 40 pg 231DNA. Panel B: 60 ng of HSD with 40 pg of 231 DNA.

Figure 1B. M-Q-MSP is specific. Primers for methylated DNA (231) do not detect unmethylated HSD (panel A), and vice versa (panel B). One ul of the 1 in 1000 dilution of the multiplex reaction was used in the Q-MSP reaction.

Table 1

RASSF1A Methylation in Ductal Carcinoma				
ID		U	M	% M
1	DCIS/ 1	81303.46	0.00	0.00
2	DCIS /1	186121.62	0.00	0.00
3	DCIS /1	7840.00	1661.54	17.49
4	DCIS /2	1221993.9	194693.08	14.00
5	DCIS/ 3	414613.88	46676.41	10.12
6	DCIS /3	3072.88	2803.76	47.71
7	DCIS /3	462237.84	121006.98	20.75
8	DCIS /3	511980.94	130374.00	20.30
9	IDC	60212.73	31235.53	34.16
10	IDC	3199.05	3121.15	49.38
11	IDC	86387.04	288.61	0.33
12	IDC	66219.05	0.00	0.00

Table 2

RASSF1A Methylation In Reduction Mammoplasty Specimens				
ID	U	M	% M	Age over 49
1	96731.95	0.00	0.00	
2	180330.70	0.00	0.00	X
3	155594.48	0.00	0.00	X
4	13343.36	0.00	0.00	
5	1792.47	0.00	0.00	
6	2103863.80	612793.20	18.00	X
7	42132.44	0.00	0.00	
8	255753.55	0.00	0.00	X
9	301421.44	0.00	0.00	X
10	9011.11	0.00	0.00	
11	342109.30	0.00	0.00	
12	185307.84	429523.60	69.00	
13	1580726.90	0.00	0.00	X
14	189218.97	0.00	0.00	X
15	9175290.00	0.00	0.00	
16	238118.89	0.00	0.00	
17	344951.38	0.77	0.00	
18	7317194.50	0.00	0.00	
19	8428731.00	0.00	0.00	
20	3189157.20	0.56	0.00	
21	1493306.80	0.00	0.00	
22	937980.30	0.00	0.00	
23	448169.25	3235.87	0.72	
24	32345.32	0.00	0.00	
25	2491771.50	0.00	0.00	
26	511569.62	0.00	0.00	
27	1032461.94	0.00	0.00	X

Table 1. By M-Q-MSP RASSF1A is frequently methylated in DCIS and invasive ductal carcinoma. Levels of RASSF1A methylation of ductal carcinoma in situ (Grade1, 2 or 3), and invasive carcinomas.

Table 2. Twenty-seven mammoplasty specimens of women with benign disease, including 19 samples of tissues from under the age of 50, and 8 samples from over the age of 50 were analyzed by M-Q-MSP. Sample#16 and Sample #12 contained 18%, and 69% values for methylated DNA levels. We are investigating the hyperplasias in these tissues more closely. Sample #23 contained less than 1% methylated alleles. No age-related methylation was observed in this small sampling.

Analysis of 28 specimens of normal peripheral blood cells showed abundant unmethylated DNA but less than 0.01% of methylated DNA. We then investigated the status of RASSF1A methylation in reduction mammoplasty specimens (Table 2).

Sample preparation and M-Q-MSP analysis. DNA will be extracted from the cytospin preparation following diagnosis and/or immunohistochemistry. A minimum of 20 breast

epithelial cells from either source is sufficient for MSP analysis. The coverslip is removed after soaking in xylene, the section is scraped and placed in 50-100 μ l of TNES with proteinase K for digestion overnight. After centrifugation at 14K, the supernatant DNA is treated with sodium bisulfite, and extracted in 10 μ l of water (1). One μ l of this is adequate for performing multiplex and Q-MSP for 6 or more genes (our unpublished work). Amplicon sizes for the seven genes vary from 70-80 bp, and all of them will be PCR-amplified under identical experimental conditions.

The contents of a typical 96 well plate consists of the following: 1) 30 patient samples, two wells per sample (one well with U or and one well with M primers), 2) two standard DNA dilution curves (dilutions of 10^{-3} , 10^{-5} , 10^{-7} , and 10^{-9} in duplicate each for U and for M primer), two genomic DNA standards of known amounts of DNA (20 ng human sperm DNA and MDA MB 231 DNA determined by OD₂₆₀) in duplicate wells, 3) two multiplex DNA standards of known % M (200/13 copies and 20,000/13 copies U/M), and water controls in duplicate from the first PCR reaction that serve as No Template Control (NTC) for each primer set U and M.

Potential Problems.

Amount of DNA available may be too low: Small quantities of DNA will be extracted from ductal lavage cells of women with DCIS and small cancers, and even less from normal individuals. We have previously successfully conducted studies on ductal cells obtained from 150 women with and without cancer. In many instances, the pathologist deemed the sample not suitable for cytology- yet we were able to obtain results by conventional MSP. To enable us to use more markers and to circumvent this problem, we have now developed the multiplex approach- with the potential to analyze 100s of genes. The quantitative feature of this assay has allowed us to set the cutoff point for normal tissue at less than 1% methylation compared to the unmethylated signal.

Sensitivity may be too low: We have successfully multiplexed 6 gene sets, and shown data for RASSF1A (Figure 1, Table 1) that look very promising. The sensitivity of our methodology is at its desired level- we are able to detect down to 40 pg of hypermethylated DNA in presence of 40 ng unmethylated DNA. Improvements by changing magnesium concentration, primer locations, and other experimental PCR conditions will be tested. We would like our marker panel to distinguish between benign and atypical hyperplasia- this experiment has never been done.

Method may detect too many positives- may not be specific: In this situation, cytology analysis will be negative and Q-MSP will be positive for the same sample. We have conducted studies on 27 mammoplasty specimens, many of which had fibrocystic changes and a few had ductal hyperplasia. Nearly all of them were negative for methylation. Thus we do not expect rampant positivity in benign disease breasts. The two positive samples appear to be those with hyperplasias, and in one case, papilloma in the contralateral breast. The information obtained from this proposal will be regarded as hypothesis generating, providing the foundation for planning a more definitive clinical trial. In particular, this information will provide the statistics necessary for appropriate power calculation to design a definitive clinical trial.

Months 12-18: Test fluid from contralateral ducts (tumor-free by mammogram and clinical exam) of 50 patients with breast cancer. Complete MSP assays on the fluid

obtained from both breasts of a total of 50 cancer patients. If recovery of cells is not satisfactory, optimize conditions, add patients to the study to get results from approximately 200-300 samples of ductal fluid (2-3 ducts per breast X 50) from 50 individuals. Compare MSP results with cytopathological data, and histopathology of the resected tumor, on each sample.

Months 18-30: Approach high-risk, tumor free women who attend the BOSS (breast ovarian surveillance service) clinic in Johns Hopkins, and other high risk individuals to undergo this procedure. Accrual will be slower in this category until the minimal discomfort involved and potential benefit becomes a publicized fact. Enter 50 individuals into the study. Perform MSP on cells obtained from each ductal lavage, on a total of approximately 200 samples.

Months 30 to 36: Complete comparison of MSP results to cytopathologic and histopathology data, and data obtained by DNA analysis of tumor tissue obtained after surgery. Write and communicate papers.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) The clinical trial has been initiated. Eleven women have undergone ductal lavage of both breasts- the tumored breast and the contralateral high-risk breast. Wash fluids have been stored. Ductal cells are being examined by the cytopathologist.
- 2) A multiplex quantitative methylation specific PCR assay has been developed for all five marker genes, that include RASSF1A and HIN-1. The sensitivity and specificity of the test have been completed.

REPORTABLE OUTCOMES:

Publications

MJ Fackler, E Evron, SA. Khan and S Sukumar. Novel agents for chemoprevention, screening methods and sampling issues. J of Mammary Gland Biology and Cancer, (in press. 2003)

Fackler MJ, Mc Veigh M, Evron E, Mehrotra J, Sukumar S, Argani P. DNA Methylation of *RASSF1A*, *HIN-1*, *RAR-α*, *Cyclin D2* and *Twist* in *In Situ* and Invasive Lobular Breast Carcinoma (manuscript submitted to International Journal of Cancer), April, 03.

Vali M, Mehrotra J, McVeigh M, Kominsky, S, Sacchi, S, Korz, D, Argani P, and S. Sukumar. Very high incidence of hypermethylated genes in breast cancer metastasis to the bone, brain and lung. (manuscript in preparation)

Mehrotra J, MM Ganpat, Y Kanaan, MJ Fackler, M McVeigh, J Lahti-Domenici, K Polyak, P Argani, E Garrett, T Naab, C Broome, and S Sukumar. Distinct DNA

methylation patterns in breast cancers among African-American and Caucasian women (manuscript in preparation, 03).

Presentations:

June 12, 2003, Howard/Hopkins Partnership Steering Committee Meeting, Howard University Cancer Center, Washington, D.C. "Comparative Gene Expression Analysis in African American and Caucasian Breast Cancer"

March 27-30, 2003, 3rd International Santa Barbara Symposium, The Intraductal Approach to Breast Cancer, Santa Barbara, California, "Detection of Breast Cancer Cells in Ductal Lavage and Blood Using Hypermethylated Gene Markers"

January 7-8, 2003, Institute of Medicine Committee on New Approaches to Early Detection and Diagnosis of Breast Cancer. Workshop on New Approaches to Breast Cancer Detection, Washington, D.C., "The Search for Breast Cancer Biomarkers"

January 22-23, 2003, National Council for Johns Hopkins Medicine, Meeting and Reception, Palm Beach, Florida, "Breast Cancer Research – Dawn of a New Era"

September 25–28, 2002, Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orange County Convention Center, Orlando, Florida, "Early Detection of Breast Cancer by Molecular Analysis of Ductal Lavage Fluid"

September 19 – 21, 2002, Mildred Scheel Cancer Conference, Frankfurt, Germany, "Gene expression profiling of breast cancer – from bench research to early detection markers"

Patent application – Aberrantly Methylated Genes as Markers of Breast Malignancy (Docket # JHU1630; Ref. # DM-3729)

CONCLUSIONS:

Cells obtained by ductal lavage will prove to be valuable resource for detecting breast cancer cells early. Recognizing the paucity of the cells, methods need to be developed that will increase the specificity and sensitivity of detection methods. This method may prove to be a PAP test for the breast.

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APPENDICES:

None.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME		POSITION TITLE	
Saraswati Sukumar, Ph.D.		Professor of Oncology/Pathology	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Nagpur University, Nagpur, India	M.S.	1969	Biochemistry
Atomic Energy Commission at Cancer Institute, Madras, India	Research Fellow	1971-76	Microbiology
Nagpur University, Nagpur, India	Ph.D.	1977	Biochemistry
National Cancer Institute, NIH, Bethesda, MD	Postdoc. Fellow	1978-83	Immunology, Molecular Biology

A. Position and Honors

- 1976-78 Assistant Research Officer, in charge of Tumor Biology Section, Indian Council of Research in Indian Medicine, Madras, India.
- 1978-83 Visiting Associate, Laboratory of Immunobiology, National Cancer Institute, Bethesda, MD (Section Chief, B.Zbar)
- 1983-88 Scientist Associate, NCI and Bionetics Research Inc., NCI/Frederick Cancer Research Facility, Frederick, MD (Section Chief, Mariano Barbacid)
- 1988-89 Senior Research Associate, Dulbecco Laboratory, The Salk Institute for Biological Studies LaJolla, CA
- 1989-94 Assistant Professor, Molecular Biology of Breast Cancer Laboratory, The Salk Institute for Biological Studies, LaJolla, CA
- 1994- Associate Professor of Oncology and Director of Basic Research, Breast Cancer Program, The Johns Hopkins University School of Medicine, Baltimore, MD
- 1996-02 Associate Professor of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD
- 2002- Professor of Oncology and Pathology, Director of Basic Research; Breast Cancer Program, The Johns Hopkins University School of Medicine, Baltimore, MD
- Scientific Counselor, National Institute of Environmental Health Sciences, Research Triangle Park, NC, 1991-94
- Member of the Board of Governors, International Association for Breast Cancer Research, 1991-94
- Member, American Cancer Society Grant Review Committee, 1992-1998

B. Selected peer-reviewed publications (out of 65)

- Sukumar S, Notario V, Martin-Zanca D, Barbacid M: Induction of mammary carcinomas in rats by nitroso-methyl-urea involves the malignant activation of the H-ras-1-locus by single point mutations. *Nature* 306:658, 1983.
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C. Research Support

Sukumar, S.

ONGOING

P50 CA88843 – Sukumar (PI) Project 1A	9/29/00-9/28/05	10%
NIH/NCI	\$177,872	

Molecular Markers for Breast Cancer

Specialized Program in Research Excellence (SPORE in Breast Cancer)

The overall goal of this proposal is to identify major molecular alterations in carcinoma of the breast by microarray analysis of all stages of tumor development.

DAMD-17-01-1-0285 - Sukumar (PI)	06/01/01-5/31/04	10%
DOD-USAMRMC	\$100,000	

Imaging the vascular and metabolic impact of claudin-7, a tight junction protein, in transgenic human breast cancer models

SPORE in Breast Cancer – Project 3C Jaffee (PI)	09/30/00 to 09/29/05	5%
NIH/NCI	\$179,678	

Vaccines: A New Paradigm for Breast Cancer Prevention

The overall goal of this proposal is to study vaccines and to use the information gained to design clinical trials aimed at both the treatment and prevention of breast cancer. The success of these studies may define a new paradigm for cancer prevention.

SPORE in Breast Cancer – Project 1B Gabrielson (PI)	09-30-00 to 09/29/05	5%
NIH/NCI	\$188,695	

Molecular phenotypes of breast cancer

The goal of this project is to distinguish biologically different group of ductal breast cancer from one another using gene expression profiles.

DAMD-17-01-1-0286 - Sukumar (PI)	6/1/01-5/31/04	15%
DOD-USAMRMC	\$100,000	

Early detection of cancer by molecular analysis of ductal lavage fluid

Five markers cyclin D2, RAR beta, Twist, RASF and HIN-1 will be evaluated in ductal cells by quantitative methylation specific PCR

National Cancer Institute, US4 - Sukumar (PI)	3/1/01-2/28/04	10%
	\$125,000	

Partnership between Howard University and Johns Hopkins/National Cancer Institute.

Comparative gene expression analysis of african american and caucasian breast cancer
microarray analysis of African American and Caucasian breast cancer stratified by ER status.

P50-CA86346-02 Bhujwalla (PI)	7/1/03- 6/30/08	5%
NIH		

Multidisciplinary functional imaging of cancer

In vivo cellular and molecular imaging centers

Pending

SPORE Supplement – Davidson 07/03 – 07/05
 Co-PI S.Sukumar \$200,000
 Surrogate Endpoints in Prevention Studies and Ductal Lavage

Avon Breast Cancer Crusade – NCI 07/03 – 06/05
 CA88843-AV-14P1 \$122,324
 Avon- - NCI Progress for Patients (PFP) Awards Program

DOD – Predoctoral Traineeship Award – BC 030941 06/04 – 06/07
 Liangfeng Han \$50,000
 Characterization of the role of HEYL in angiogenesis and breast cancer Development

DOD – Predoctoral Traineeship Award 06/04 – 06/07
 Theresa Swift-Scalan, M.S., R.N. \$50,000
 Epigenetic Modifiers in Breast Cancer Risk

DOD – Postdoctoral Award – BC 030470 06/04 – 06/07
 Xinyan Wu, Postdoctoral Fellow \$100,000
 Role of HOXA5 in Breast Cancer Metastasis

Supplement to SPORE – CA88843 09/01/03-08/31/04
 (Administrative Supplement for equipment) \$128,186

Completed in the Last 3 Years

R01 CA48493 - Sukumar (PI) 7/1/96-6/30/01
 NIH/NCI

Role of tumor suppressor genes in chemical carcinogenesis
 This project aims at determining if expression of abundant edited Wilms' tumor suppressor gene, WT1 mediates tumorigenesis.

DAMD-17-99-1-9242 - Sukumar (PI) 9/1/99-8/31/02
 DOD-USAMRMC

Prevention of Breast Cancer by Targeted Disruption of Breast Epithelial Cells.
 The goal of this project is to determine chemical ablation of the mammary epithelium can prevent breast cancer. The hypothesis will be tested using TGF α -PE toxin in a rat mammary tumor model.

P20-CA86346-02 Bhujwalla (PI) 7/1/01- 6/30/02
 NIH

Multidisciplinary functional imaging of cancer
 Planning grants: In vivo cellular and molecular imaging centers

Susan G. Komen Breast Cancer Foundation-Sukumar (PI) 9/30/00-9/29/02
 SGKf - BCTR-2000-577
 Early Detection of Breast Cancer by Molecular Assessment of Ductal Lavage Fluid
 Three markers cyclin D, RAR- β 2 and ER will be evaluated

BCRF – Davidson (PI) 10/01/01-09/30/02
 Breast Cancer Research Foundation
 To support pre-clinical studies in breast cancer research at Johns Hopkins